1.2.4. HUMAN INSULIN PRODUCED BY RECOMBINANT METHODS

Human insulin was the first animal protein made in bacteria in a sequence identical to that of the human pancreatic peptide (Watson et al., *Recombinant DNA--A Short Course;* Scientific American Books, W. H. Freeman Co., New York, 1983, pp. 231-235). The first successful expression of human insulin in laboratory was announced in 1978 and human insulin was approved as a therapeutic drug in 1982 (Johnson, *Science*, 1983, 219:632-637).

1.2.4.1. TWO-CHAIN METHOD

According to this method, each insulin chain is produced as a β-galactosidase (β-gal) fusion protein in separate fermentations using *E. Coli* transformed with plasmids containing a DNA sequence encoding the A or B chain of human insulin, respectively. The products are intracellular and appeared in prominent cytoplasmic inclusion bodies (Williams et al., *Science*, 1982, 215(5):687-689). Recombinant proteins produced in *E. Coli* usually represent 10-40% of the total protein (Burgess, *Protein Engineering;* Oxender. D.L., Fox, C.F., Eds.; Alan R. Liss, Inc.; New York, 1987; pp. 71-82.).

Once removed from the inclusion bodies, chemical cleavage by CNBr at the Met residue between the β-galactosidase and the A or B chain, followed by purification, gave separate A and B peptides. The peptides are then combined and induced to fold at a ration of 2:1 of A-B chain (S-sulfonated forms) in the presence of limited amounts of mercaptan in order to obtain an active hormone (Chance et al., *In Peptides: Synthesis-Structure-Function,* Rich D. M. Gross, E., Eds., Pierce Chemical Co., Rockford, II 1981, pp.721-728; Frank and Chance, *In Quo Vadis? Therapeutic Agents Produced by Genetic Engineering*, Joyesuk et al., Eds., Sanoff Group, Toulouse-Labege, France, 1985, pp. 137-148). After 24 h, the yield is approximately 60% based on the amount of B chain used (Chance et al.. *In Insulins, Growth Hormone and Recombinant DNA Technology,* Raven Press, New York. 1981, pp. 71-85; Johnson, *Fluid Phase Equilib.*, 1986, 29:109-123). Goeddel et al., *Proc. Natl. Acad. Sci. U.S.A.*, 1979, 76(1):106-110, obtained similar results with 20% of the total cellular protein expressed as either the A or B chain fusion protein. Subsequent folding of S-sulfonated chains give 50-80% correct folding.

The large size of the β-gal fusion protein limits yields since the fusion protein of β-gal (~1000 amino acids) and insulin A or B chain (21 or 30 amino acids, respectively) became detached from the cell's ribosome (premature chain termination during translation) and therefore yields incomplete insulin peptides (Burnett, *Experimental Manipulation of Gene Expression*, Inouye, Ed., Academic Press, New York, 1983, pp. 259-277; Hall,

Invisible Frontiers--The Race to Synthesize a Human Gene, Atlantic Monthly Press, New York, 1987). A key improvement to this approach is the use of the tryptophan (*Trp*) operon in place of the lac operon (β-gal system) to obtain a smaller fusion protein. The *Trp* operon consists of a series of five bacterial genes which sequentially synthesize the enzymes responsible for the anabolism of tryptophan. One of these enzymes, *Trp E*, has only 190 amino acids compared to β-gal's 1000 amino acids. The *Trp E* gene followed by genes for the A or B chains of insulin has the added advantage of enhancing fusion protein production from 5-10% to 20-30% of the total protein (Hall, *Invisible Frontiers--The Race to Synthesize a Human Gene*, Atlantic Monthly Press, New York, 1987) since the *Trp* promoter is a strong promoter in *E. Coli*. This leads to at last 10-fold greater expression of polypeptide when compared to the *lac* (i.e., β-gal) system (Burnett, *Experimental Manipulation of Gene Expression*, Inouye, Ed., Academic Press, New York, 1983, pp. 259-277). The *Trp* operon is turned on when the *E. Coli* fermentation runs out of tryptophan (Hall, *Invisible Frontiers--The Race to Synthesize a Human Gene*, Atlantic Monthly Press, New York, 1987: Etienne-Decent, *In Genetic Biochemistry: From Gene to Protein*, Ellis Horwood Limited, Chichester, U.K., 1988, pp. 125-127). This characteristic is beneficial during fermentation since cell mass can first be maximized. Then, when appropriate, the cell's insulin production system can be turned on by allowing the fermentation media to become depleted in Trp.

After fermentation is completed, the cells are recovered and disrupted. The cell debris is than separated from the inclusion bodies, and the inclusion bodies are dissolved in a solvent, although specifics are not known (Wheelwright, *Protein Purification,* Oxford University Press; New York, 1991, p. 217). Inclusion bodies are sometimes dissolved in 6 M guanidine HCl and 0.1 mM dithiothreitol (Burgess, *Protein Engineering,* Oxender and Fox, Eds., Alan R. Liss, Inc., New York, 1987, pp. 71-82). Next, the Trp-LE-Met-A chain and the Trp-LE-Met-B chain undergo a CNBr cleavage to release the A and B insulin chains. Further modifications of the A and B chains include oxidative sulfitolysis, purification and combination to produce crude insulin. This crude insulin is subjected to ion exchange, size exclusion, and reversed-phase high-performance liquid chromatography (RP HPLC) to produce the purified recombinant human insulin (Frank and Chance, *Munch Med. Wschr,* 1983, 125(Suppl. 1):514-520).

1.2.4.2. PROINSULIN METHOD (INTRACELLULAR)

Human insulin can also be made with recombinant microorganisms that produce intact proinsulin instead of the A or B chains separately (Kroeff et al., *J. Chromatogr,* 1989. 481:45-61). Initially, mRNA is copied into cDNA, and a methionine codon is chemically

synthesized and attached to the 5' end of the proinsulin cDNA. The cDNA is inserted into a bacterial gene in a plasmid vector that is introduced and then grown in *E. Coli*. Proinsulin can be released from the bacterial enzyme (β-gal) fragment (or alternatively from the Trp-LE/Met Proinsulin (Trp proinsulin) by destroying the methionine linker. The proinsulin chain is subjected to a folding process to form the correct intramolecular disulfide bridges, and the C peptide can then be cleaved with enzymes to yield human insulin (Frank and Chance, *Munch Med. Wschr.*, 1983, 125(Suppl. 1):514-520). In comparison, the two-chain method previously described is more complex.

Dorschug et al. constructed recombinant plasmid encoding fusion proteins containing a mini-proinsulin (B-Arg-A), expressed the fusion proteins in *E.coli* (inclusion body) and yeast (secreted), prepared correctly folded mini-proinsulin via BrCN cleavage and oxidative sulfitolysis, and converted the correctly folded mini-proinsulin into human insulin by treatment with trypsin and carboxypeptidase B (EP 0,347,781 B1; IL 9,562,511 B and AU 611,303 B2).

Tottrup and Carlsen, *Biotechnol. Bioeng,* 1990, 35:339-348 used the yeast system in an optimized batch-fed fermentation, yields of the fusion protein of superoxide dismutase-human proinsulin (SOD-PI) were reported to be 1500 mg/L. SOD-PI would be the starting material for the production of recombinant human insulin; yields of the final product have not been reported.

Recently, Castellanos-Serra et al., *FEBS Letters*, 1996, 378:171-176 expressed in *E. Coli* a proinsulin fusion protein carrying a modified interleukin-2 N-terminal peptide (1-22 amino acid residues) linked to the N-terminus of proinsulin by a lysine residue. The chimeric proinsulin was isolated from inclusion bodies, refolded via oxidative sulfitolysis, and then converted into the correctly fusion proteins insulin by prolonged reaction with trypsin and carboxypeptidase B. The IL2-proinsulin fusion can be folded correctly without first removing the IL2 fragment, thus eliminating the cyanogen bromide and the associated purification steps. However, the step of oxidative sulfitolysis and the associated purification steps cannot be avoided by the use of IL2-proinsulin fusion protein.

1.2.4.3. PROINSULIN METHOD (SECRETED)

Villa-Komaroff et al., *Proc. Natl. Acad, Sci. U.S.*, 1978, 75(8):3727-3731 were first to describe a secretion system for human proinsulin in *E. Coli*. Thim et al. constructed recombinant plasmids encoding fusion proteins containing a modified yeast mating factor α1 leader sequence and an insulin precursor (Thim et al., *Proc. Natl. Acad. Sci. USA*, 1986, 83:6766-6770). The leader sequence serves to direct the fusion protein into the secretory pathway of the yeast cell and to expose the fusion protein to the Lys-Arg